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BRIEF REPORT

Two novel variants of the *ABCG5* gene cause xanthelasmas and macrothrombocytopenia: a brief review of hematologic abnormalities of sitosterolemia

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Essentials

- Diagnosis of sitosterolemia, a rare recessive or syndromic disorder, is usually delayed.
- Peripheral blood smear is extremely useful for establishing the suspicion of sitosterolemia.
- High-throughput sequencing technology enables the molecular diagnosis of inherited thrombocytopenias.
- Accurate characterization of sitosterolemia helps us determine appropriate management.

Summary. *Background:* Sitosterolemia (STSL) is a recessive inherited disorder caused by pathogenic variants in the *ABCG5* and *ABCG8* genes. Increased levels of plasma plant sterols (PSs) usually result in xanthomas and premature coronary atherosclerosis, although hematologic abnormalities may occasionally be present. This clinical picture is unfamiliar to many physicians, and patients may be at high risk of misdiagnosis. *Objectives:* To report two novel *ABCG5* variants causing STSL in a Spanish patient, and review the clinical and mutational landscape of STSL. *Patient/Methods:* A 46-year-old female was referred to us with lifelong macrothrombocytopenia. She showed familial hypercholesterolemia-related xanthomas. Molecular analysis was performed with high-throughput sequencing. Plasma PS levels were evaluated with gas–liquid chromatography. The STSL landscape was reviewed with respect to specific online databases and all reports published since 1974. *Results:* A blood smear revealed giant platelets and stomatocytes. Novel compound heterozygous variants were detected in exons 7 (c.914C>G) and 13 (c.1890delT) of *ABCG5*. The patient showed an increased plasma level of sitosterol. These findings support the diagnosis of STSL. In our review, we identified only 25 unrelated STLS patients who presented with hematologic abnormalities including macrothrombocytopenia. It remains unknown why only some patients develop hematologic abnormalities. *Conclusions:* This is the first Spanish STSL patient to be reported and molecularly characterized. The early diagnosis of STLS is strongly supported by the presence of stomatocytes in blood smears. The definitive diagnosis of STSL by measurement of serum PS levels and molecular analyses prompted the use of ezetimibe therapy.

Keywords: blood platelet disorders; genetic testing; high-throughput nucleotide sequencing; sitosterols; thrombocytopenia.

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Introduction

Sitosterolemia (STSL), also known as phytosterolemia, is a rare autosomal recessive inherited sterol storage disorder characterized by increased levels of plasma plant

sterols (PSs), including stigmasterol, campesterol, and, most abundantly, sitosterol [1]. STSL is caused by pathogenic variants in the genes of two adjacent ATP-binding cassette subfamily G members, i.e. members 5 and 8 (*ABCG5* and *ABCG8*), which encode sterolin-1 and sterolin-2, respectively [2]. These proteins, which contain six transmembrane domains and an ATP-binding domain, travel as heterodimers to the apical membrane of the enterocyte and the canalicular membrane of the biliary tract, where they become full, active transporters of sterols, although they require ATP to function [2,3]. Sterolins regulate the network of absorption and excretion of sitosterol and cholesterol. In enterocytes, they promote the flow of sitosterol back into the intestinal lumen and liver, and the excretion of sterols into bile [3]. Like those of other inherited thrombocytopenias (ITs), the prevalence of STSL is unknown, although one Asian individual with STSL was identified incidentally out of 2542 persons [4]. Clinical features of STLS usually include tendinous and cutaneous xanthomas, premature coronary atherosclerosis and derived complications, and arthritis or arthralgia [5]. These characteristics are shared with familial hypercholesterolemia (FH), so STSL may be misdiagnosed as homozygous FH, especially in pediatric patients [6,7]. Unlike patients with FH, patients with STSL usually respond well to a low-cholesterol diet and/or bile acid sequestrants such as colestimide and colestyramine [6]. Hematologic abnormalities, e.g. hemolytic anemia (HA), iron deficiency anemia, macrothrombocytopenia (i.e. large platelets), abnormal bleeding, and/or splenomegaly, may coexist with other clinical features [8]. These hematologic problems have been occasionally reported as the only clinical symptoms in otherwise asymptomatic STSL patients [5,8]. Combined macrothrombocytopenia and stomatocytosis, known as Mediterranean stomatocytosis/macrothrombocytopenia, was initially described in Mediterranean migrants to Australia [9]. The diagnosis of STSL is usually delayed because many physicians are unfamiliar with its clinical picture [5]. This delay is important because, like patients with other ITs, non-diagnosed patients may be at high risk of receiving inappropriate treatment for a considerable period [8,10].

We report two novel *ABCG5* variants causing syndromic macrothrombocytopenia in a Spanish family, in a case previously reported to be one of FH. We summarize the clinical and mutational landscape of STSL in relation to hematologic abnormalities, and propose some clinical indications for a PS assay and for molecular analysis.

Patients and methods

The propositus was a 46-year-old female referred to us with lifelong macrothrombocytopenia. Her medical history included palpebral surgery for xanthelasma on several occasions, and clinical suspicion of FH. Her paternal grandparents were first cousins. The patient suffered from

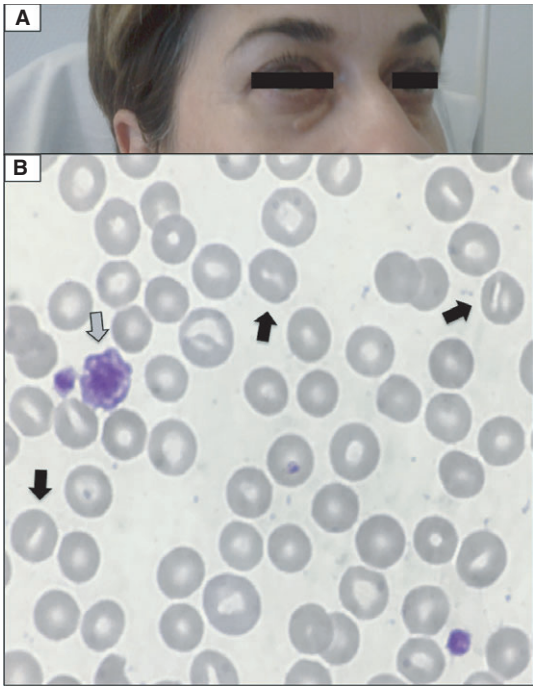

Fig. 1. Xanthelasma and peripheral blood film findings in the sitosterolemia patient. (A) Xanthelasma in the eyes. (B) Peripheral blood smear revealed stomatocytes (black arrows) and macrothrombocytes (gray arrow), consistent with hereditary sitosterolemia. [Color figure can be viewed at wileyonlinelibrary.com]

arthralgia and mild ecchymosis after trauma. Examinations revealed no hepatosplenomegaly or clinically significant lymphadenopathy, but she presented with xanthelasmas (Fig. 1A). Bleeding was of minor relevance, with an ISTH Bleeding Assessment Tool score of 1. Analysis of blood parameters showed the following: hemoglobin level, 13.3 g dL^{-1}; reticulocytes, 2%; platelet count, 69.8×10^3 mm^{-3}. The mean platelet volume was 15.5 fL (Sysmex XE 2100; Roche, Basel, Switzerland). No biochemical abnormalities were found. Peripheral blood (PB) smears revealed abnormally shaped erythrocytes (22% stomatocytes) and giant platelets, some as large as the erythrocytes (Fig. 1B). Platelet aggregation in response to ADP and ristocetin was normal. The levels of platelet glycoprotein (GP) Ib–IX and GPIIb–IIIa measured by flow cytometry were also normal (results not shown).

Given the syndromic macrothrombocytopenia, the patient's DNA was screened for molecular variants in 71 candidate genes related to inherited platelet disorders (IPDs) by use of an established high-throughput sequencing (HTS) platform (MiSeq; Illumina, San Diego, CA, USA) and accompanying variant analysis [10,11]. Serum PS and cholesterol levels were quantified with gas–liquid chromatography (GLC) [3]. Briefly, PSs and cholesterol were isolated following alkaline hydrolysis and extraction with organic solvent, and then analyzed with GLC in an Agilent 7890 chromatograph fitted with a flame detector (trimethylsilyl derivative; BSTFA 1%/TMCS in pyridine;

Sigma, St. Louis, MO, USA) in a DB1701 (S&W Scientific, Folsom, CA, USA) column. Epi-coprostanol (Sigma) was used as the internal standard. Reference values were from individuals aged 1–60 years whose cholesterol metabolism was unaltered. The normal ranges (NRs) for sitosterol, campesterol, β-cholestanol and cholesterol were: < 10 μM, < 3 μM, 2.2–12.6 μM, and 1.7–6.6 mM, respectively.

The study was performed in accordance with the Declaration of Helsinki. The patient and healthy controls provided written informed consent.

Results and discussion

We report the clinical and molecular features, and treatment outcome, of a patient presenting with xanthelasmas who had been previously misdiagnosed with FH. Notably, her blood film showed prominent stomatocytes and giant platelets (Fig. 1). It should be emphasized that careful examination of the PB smear has been proposed as the most important aspect of the diagnostic approach to this disorder [12,13]. Some authors have also suggested that platelet size should be reviewed in all patients with hypercholesterolemia [5]. HA and thrombocytopenia are hematologic abnormalities that are commonly encountered in daily practice; the coexistence of unexplained HA with stomatocytosis and macrothrombocytopenia should alert physicians to the possibility of STSL [14]. Nevertheless, these STSL-associated hematologic abnormalities are still not recognized by most physicians, and many STSL patients are not diagnosed until adulthood [12,15]. In addition, STSL patients may suffer from long-term misdiagnosis of immune thrombocytopenia (ITP), Evans syndrome, or ITP secondary to arthritis. Consequently, many STSL patients are treated with useless or even harmful therapies, such as steroid therapy and/or splenectomy, as occurs with other misdiagnosed ITs [10,12,15]. For the molecular analysis of this patient, we used a novel HTS panel to simultaneously process a large number of genes involved in IPDs [10,11]. The HTS test revealed novel compound heterozygote variations affecting exons 7 (c.914C>G; p.T305R) and 13 (c.1890delT; p.F630L fs8X) of *ABCG5*. As routine cholesterol measurement methods do not discriminate cholesterol from PSs, we used a specific GLC method [3,12] to determine cholesterol and PS levels separately. The patient's cholesterol level was 2.7 mM, which was in the NR, owing to her previous treatment with statins. Measurements of plasma PS levels revealed high concentrations of sitosterol (668.2 μM), campesterol (169.6 μM), and β-cholestanol (30.7 μM). These findings confirmed the diagnosis of STSL in our patient. There was a striking delay of decades between the onset of symptoms and the STLS diagnosis. Long delays in providing a definitive diagnosis have also been reported in other cases of STLS [12]. Such delays are not inconsequential, as they can lead to inappropriate clinical management, including delayed PS restriction and administration of sterol absorption inhibitor [6], and a subsequent risk of advanced atherosclerotic cardiovascular disease and poorer clinical outcome in affected patients. Following her definitive STLS diagnosis, our patient began ezetimibe therapy, which effectively decreased the plasma levels of total cholesterol and LDL cholesterol [16]. Although ezetimibe has been shown to improve thrombocytopenia in *ABCG5*-knockout mice, this benefit has not been consistently found in humans [17,18]. After 4 months of ezetimibe treatment, sitosterol levels were reduced by 30% (473.1 μM), whereas cholesterol (2.4 mM), campesterol (156.3 μM) and β-cholestanol (23 μM) levels remained similar. However, our patient still showed moderate macrothrombocytopenia (platelet count: 80.9×10^3 mm^{-3}).

Since the first description of this disorder in 1974, < 100 related cases have been found worldwide [1,4,12]. To our knowledge, only 25 unrelated STSL patients presenting with hematologic abnormalities at the time of diagnosis have been reported, some of whom had been previously misdiagnosed with ITP or HA, and had therefore undergone splenectomy (Table 1). Most of these described families were consanguineous. Although consanguinity was detected in this pedigree, the patient had a compound heterozygosis, so the family relationship is unlikely to be relevant here (Fig. 2A). The mechanism by which sitosterol accumulation might negatively affect platelet structure and function is not well understood. Whereas some authors have described decreased platelet activation responsiveness in animal models of sitosterolemia [19], others have identified platelet hyperactivation, reduced $\alpha_{IIb}\beta_3$ surface expression, loss of GPIba–FlnA linkage, and microparticle formation [17]. As in previous STLS cases (Table 1) [12], our patient showed almost negligible bleeding diathesis and no platelet defects with respect to aggregation or the expression of major GPs.

The molecular pathology involved in STSL affects *ABCG5* and *ABCG8* [20,21]. These two genes probably evolved from a common ancestral gene by a tandem duplication and inversion event. The intergenic region between *ABCG5* and *ABCG8* is very small (< 160 bases) and contains no conserved TATA motif(s), suggesting that transcriptional factors help to regulate this locus [22]. Moreover, mutational founder effects underlie many of the cases, suggesting that this disease has been present for many generations, perhaps for > 4000 years [23]. However, despite the close proximity and homology of *ABCG5* and *ABCG8*, considerably more polymorphisms are present in *ABCG8* than in *ABCG5* [23]. Caucasians (mostly of northern European origin) frequently carry mutations in *ABCG8*, whereas Chinese, Japanese and Indian patients often have mutations in *ABCG5* (Table 1) [6]. Among these, 35% and 65% feature homozygous or compound heterozygous variants in *ABCG5* and *ABCG8*, respectively [6,24]. Some heterozygotes showed higher

Table 1 Clinical characteristics, laboratory findings and molecular variants in sitosterolemia patients who showed macrothrombocytopenia

References	Ethnicity	Age (years)/ sex	Consanguinity	Gene	Gene variant	Inherited pattern	Bleeding	Splenomegaly	Hematologic features	Other
[14]	Turkish	12/M	Yes	*ABCG5*	IVS10-1G>T	Homzygous	Epistaxis	Yes	HA and stomatocytes Giant latelet count (55×10^3 mm^{-3})	Growth retardation
[30]	Caucasian	47/M	No	–	–	–	No	Yes	HA and stomatocytes Giant platelet count (85×10^3 mm^{-3})	Xanthomas Cholelithiasis Splenectomy
[21]	Asian	56/F	Yes	*ABCG5*	p.F283S fs5X (19-bp insertion)	Homozygous	No	No	Giant platelet count (98×10^3 mm^{-3})	Xanthomas Arthritis
[21]	Asian	32/F	No	*ABCG5*	p.F283S fs5X p.R446X	Compound heterozygous	No	No	HA and stomatocytes Giant platelet count (70×10^3 mm^{-3})	Xanthomas Arthritis
[7]	Caucasian	8/F	No	–	–	Compound heterozygous	No	No	Giant platelet count (96×10^3 mm^{-3})	Xanthomas FHC
[5]	Asian	15/F	Yes	*ABCG5*	p.E77X	Homozygous	No	Yes	HA and stomatocytes Giant platelet count (98×10^3 mm^{-3}) LTA: reduced ristocetin	Growth retardation Three other affected family members
[5]	Caucasian	11/M	No	*ABCG5*	p.E146X IVS11+3insT	Compound heterozygous	Epistaxis	Yes	HA and stomatocytes Giant platelet count (102×10^3 mm^{-3}) LTA: reduced ristocetin	One other affected family member
[5]	Caucasian	29/M	No	*ABCG8*	p.Q271X IVS8-1G>A	Compound heterozygous	No	Yes	HA and stomatocytes Giant platelet count (108×10^3 mm^{-3}) LTA: reduced ristocetin	One other affected family member
[5]	Caucasian	14/F	No	*ABCG8*	p.W361X	Homozygous	No	Yes	HA and stomatocytes Giant platelet count (169×10^3 mm^{-3}) LTA: reduced ristocetin	Deafness
[5]	Caucasian	26/F	No	*ABCG8*	p.W361X	Homozygous	Postsurgery	Yes	HA and stomatocytes Giant platelet count (137×10^3 mm^{-3}) LTA: reduced ristocetin	Xanthomas FHC
[29]	Asian	24/F	Yes	*ABCG5*	p.Q22X	Homozygous	Epistaxis Gingival Menorrhagia	Yes	HA and stomatocytes Giant platelet count (56×10^3 mm^{-3}) LTA: reduced ristocetin	Xanthomas Cholelithiasis Two other affected family members Splenectomy
[13]	–	28/F	No	–	–	–	No	No	HA and stomatocytes Giant platelet count (77×10^3 mm^{-3})	No

Table 1 (Continued)

References	Ethnicity	Age (years)/ sex	Consanguinity	Gene	Gene variant	Inherited pattern	Bleeding	Splenomegaly	Hematologic features	Other
[24]	Asian	–	–	*ABCG5*	p.R446X	Homozygous	No	Yes	HA and stomatocytes Macrothrombocytopenia	Xanthomas
[24]	Asian	–	–	*ABCG8*	del43683-43724	Homozygous	No	Yes	HA and stomatocytes Macrothrombocytopenia	Xanthomas
[24]	Asian	–	–	*ABCG8*	del1938C-1939G/ ins1938T	Homozygous	No	Yes	HA and stomatocytes Macrothrombocytopenia	Xanthomas
[12]	Asian	25/F	No	*ABCG5*	p.Q22X	Homozygous	Mild	Yes	HA and stomatocytes Giant platelet count (36.1×10^3 mm^{-3})	Xanthomas Two other affected family members
[12]	Asian	34/F	No	*ABCG5*	p.R446X	Homozygous	Moderate	Yes	HA and stomatocytes Giant platelet count (56×10^3 mm^{-3})	Xanthomas Cholelithiasis Abnormal liver test
[12]	Asian	43/F	No	*ABCG8*	p.M614-K628del p.V642S fs20X	Compound heterozygous	Mild	Yes	HA and stomatocytes Giant platelet count (20×10^3 mm^{-3})	Xanthomas CHD Short stature Fibroma
[12]	Asian	61/M	No	*ABCG5*	p.R446X IVS9+2A>G	Compound heterozygous	Mild	Yes	HA and stomatocytes Giant platelet count (21×10^3 mm^{-3})	No Four other affected family members
[12]	Asian	31/M	No	*ABCG5*	p.R419H	Homozygous	Moderate	Yes	HA and stomatocytes Giant platelet count (12.8×10^3 mm^{-3})	No
[12]	Asian	58/M	No	*ABCG8*	p.L86P fs99X	Homozygous	No	Yes	HA and stomatocytes Giant platelet count (20.6×10^3 mm^{-3})	Xanthomas Cholelithiasis Abnormal liver test
[12]	Asian	45/F	No	*ABCG5*	IVS7+2G > A	Homozygous	No	Yes	HA and stomatocytes Giant platelet count (30×10^3 mm^{-3})	Xanthomas
[12]	Asian	60/F	No	*ABCG8*	p.R263Q p.E500D fs104X	Compound heterozygous	No	Yes	HA and stomatocytes Giant platelet count (59×10^3 mm^{-3})	Arthralgia CHD
[27]	Caucasian	59/F	No	*ABCG8*	p.Q302X	Homozygous	No	No	HA and stomatocytes Macrothrombocytopenia	Xanthelasmas CHD
[24]	Asian	31/M	Yes	*ABCG5*	p.R419H	Homozygous	Moderate	Yes	HA and stomatocytes Giant platelet count (15×10^3 mm^{-3})	Xanthomas CHD Arthritis Splenectomy

CHD, coronary heart disease; F, female; FHC, familial hypercholesterolemia; HA, hemolytic anemia; LTA, light transmission aggregometry; M, male.

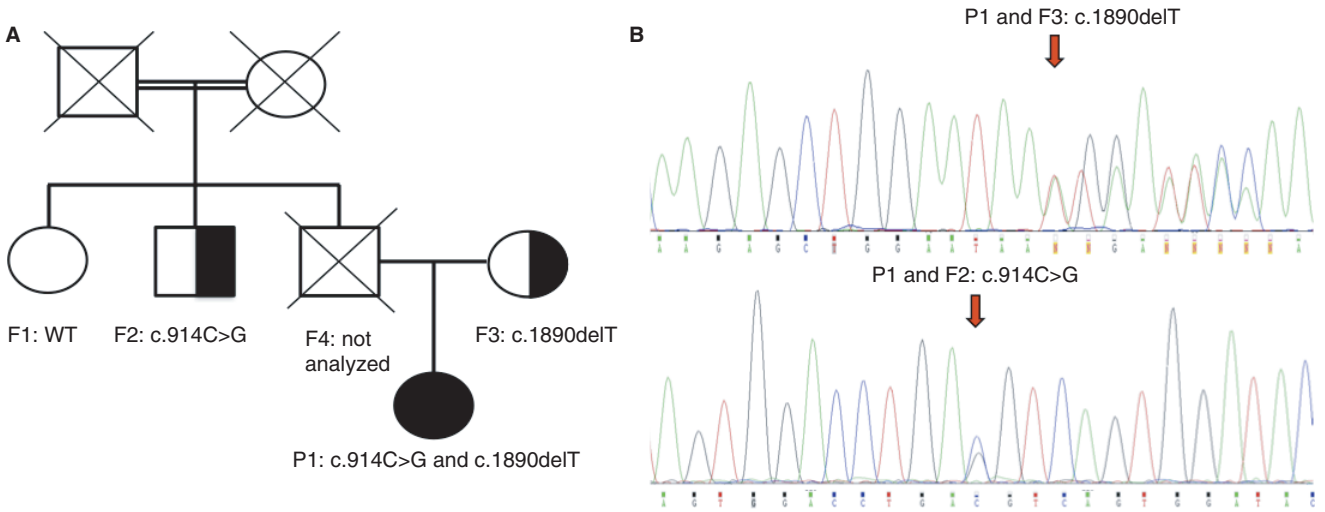

Fig. 2. Family pedigree and chromatogram showed the variants in *ABCG5*. (A) Family pedigree. The propositus (P1) was compound heterozygous (c.914G>C and c.1980delT). Heterozygous family members are shown as half-filled symbols. The father (F4) was deceased, and therefore could not be analyzed. (B) Chromatogram: the proband (P1) showed compound heterozygosity for c.914G>C and c.1980delT, whereas F2 and F3 probands were only heterozygous for c.914G>C and c.1980delT, respectively. WT, wild type. [Color figure can be viewed at wileyonlinelibrary.com]

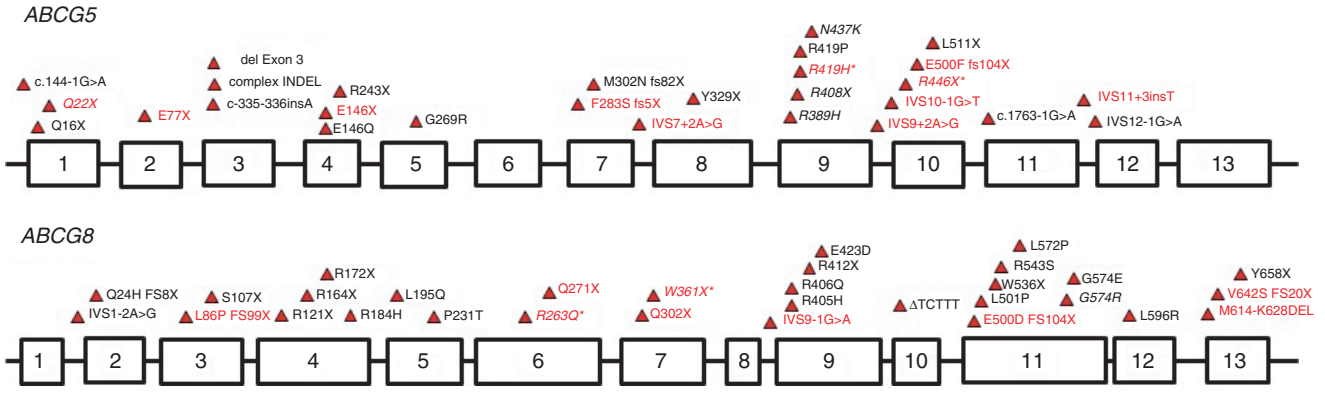

Fig. 3. Molecular landscape of variants reported in *ABCG5* and *ABCG8*. The structures of *ABCG5* and *ABCG8*, comprising 13 exons, are shown. Reported variants are indicated above each gene structure. Red symbols indicate patients with macrothrombocytopenia, and italics indicate recurrent variants (i.e. appearing in more than one patient). Asterisks (*) indicate genetic variants associated with hematologic abnormalities in some patients. [Color figure can be viewed at wileyonlinelibrary.com]

than normal PS levels, but these were still substantially lower than those in homozygotes [25].

Twenty-eight variants have previously been reported in the *ABCG5* gene (Table 1; Fig. 3), and only six variants are present in more than one patient. Several of these are nonsense variants (eight) or frameshift variants (three), or affect the splicing site (eight) or code for truncated polypeptides (two). Others are missense variants (seven) that interfere with the formation of stable ABCG5–ABCG8 heterodimers and their trafficking out of the endoplasmic reticulum [6,26–29] (Fig. 3).

Only 11 of the 28 reported variants are associated with macrothrombocytopenia (Fig. 3). The patient reported here carries two variants affecting exons 7 and 13 of the *ABCG5* gene. The former, c.914C>G; p.T305R, a rare single-nucleotide polymorphism (SNP) (rs143740796) with a minor allele frequency of 0.00002486 (http://exac.broadinstitute.org), is predicted to be pathogenic by *in silico* models (SIFT, POLYPHEN-2, MUTATIONASSESSOR, and MUTATIONTASTER). This SNP affects highly conserved residues at the N-terminal domain, in the intracytoplasmic region of the ABCG5 protein [3]. The remarkable degree of conservation of the STSL locus in fish, amphibians, rodents and humans suggests that the polymorphic changes may have a dramatic effect on function [3]. The second genetic variant in the patient is a novel microdeletion in exon 13 (c.1890delT; p.F630L fs8X) that causes replacement of phenylalanine by leucine at amino acid position 630, producing a frameshift and stop codon at amino acid 638 (Fig. 2B).

The heterogeneous landscape of STSL may be related both to other genetic factors and to environmental conditions, which can modify gene expression or sterol absorption [15]. For example, an olive oil-rich diet contributes to elevated PS levels in blood [15]. Phenotypic heterogeneity could also arise by gene conversion that restores the wild-type sequence of the maternal or the paternal allele, converting the compound heterozygous condition to a simple heterozygous one, in the form of mosaicism [26].

The underlying mechanism responsible for the hematologic abnormalities observed in some patients with STSL is most likely the accumulation of circulating PSs in blood cell membranes, leading to their abnormal morphology and function [29]. Thus, excess PS levels promote membrane stiffness and predispose to rupture. Likewise, PS enrichment in platelet membranes might affect their size, number, and function, which could be related to bleeding episodes [29]. These hypotheses were suggested to explain the behavior of ABCG5-deficient and ABCG8-deficient mice [17,19]. In humans, however, only some patients with STSL show hematologic abnormalities. Moreover, some variants, such as R419H and R446X (*ABCG5*), are associated with thrombocytopenia, although not in all patients, implying that unknown mechanisms are also involved (Table 1). Our screening of 71 genes related to IPD yielded no candidate variants other than those mentioned above. It has been suggested that whole-exome or whole-genome analysis might identify new genetic determinants that predispose patients to having these clinical conditions.

In summary, we have characterized the first case of STSL in a Spanish patient by using a novel HTS multigenic platform for an initial molecular survey. A definitive diagnosis of STSL based on the measurement of serum PS levels prompted the use of ezetimibe therapy, which effectively reduced serum PS levels. It is essential to suspect and correctly diagnose this controllable disorder to ensure its proper management, and prevent cardiovascular diseases.

Addendum

R. Benito, K. Janusz, and J.M. Hernández-Sánchez undertook the molecular studies. J. M. Bastida, S. Marcellini, M. Díez-Campelo, A. Hortal, M. Girós, J. Rivera, and M. L. Lozano interpreted the clinical and laboratory data, blood smears, PS plasma levels, platelet aggregation, and flow cytometry. J. M. Bastida and R. Benito interpreted the molecular analyses. J. M. Bastida wrote the manuscript. J. M. Hernández-Rivas, M. L. Lozano, J. Rivera, and J.R. González-Porras critically reviewed the manuscript. J. M. Hernández-Rivas was the principal investigator. All authors read and approved the final manuscript.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interest.

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